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(54) Title: METHODS FOR INDUCING T CELL NON-RESPONSIVENESS TO A TISSUE OR ORGAN GRAFT

(57) Abstract: Methods for inducing T cell tolerance to a tissue or organ graft in a transplant recipient are disclosed. The methods involve administering to a subject antagonists of the receptor which inhibit interaction of the ligand with the receptor, and CD8+ T cell depleting agents. In a preferred embodiment, the molecules on the surface of the T cell which mediate contact-dependent helper effector function are CD154. Preferred CD154 antagonists and CD8+ T cell depleting agents are anti-CD154 and CD8 antibodies. The CD154 antagonists and CD8+ T cell depleting agents are typically administered to a transplant recipient following transplantation of the tissue or organ. The methods of the invention can be used to induce T cell tolerance to transplants such as pancreas, pituitary, liver, kidney, heart, lung, skin, muscle, brain tissue, nerve tissue, stomach, intestines, Adenosine Deaminase-expressing cells, and leptin-expressing cells. A method for treating a skin disorder comprising administering to a subject allogeneic or xenogeneic tissues, organs, or cells expressing donor antigens, CD154 antagonists and CD8+ T cell depleting agents and allogeneic skin graft cells is also disclosed.

WO 01/00679 A2

METHODS FOR INDUCING T CELL NON-RESPONSIVENESS TO A TISSUE OR ORGAN GRAFT

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Background of the Invention

To induce antigen-specific T cell activation and clonal expansion, two signals provided by antigen-presenting cells (APCs) must be delivered to the surface of resting T lymphocytes (Jenkins, M. and Schwartz, R. (1987) *J. Exp. Med.* 165, 302-319; Mueller, D.L., et al. (1990) *J. Immunol.* 144, 3701-3709; Williams, I.R. and Unanue, E.R. (1990) *J. Immunol.* 145, 85-93). The first signal, which confers specificity to the immune response, is mediated via the T cell receptor (TCR) following recognition of foreign antigenic peptide presented in the context of the major histocompatibility complex (MHC). The second signal, termed costimulation, induces T cells to proliferate and become functional (Schwartz, R.H. (1990) *Science* 248, 1349-1356). Costimulation is neither antigen-specific, nor MHC restricted and is thought to be provided by one or more distinct cell surface molecules expressed by APCs (Jenkins, M.K., et al. (1988) *J. Immunol.* 140, 3324-3330; Linsley, P.S., et al. (1991) *J. Exp. Med.* 173, 721-730; Gimmi, C.D., et al., (1991) *Proc. Natl. Acad. Sci. USA.* 88, 6575-6579; Young, J.W., et al. (1992) *J. Clin. Invest.* 90, 229-237; Koulova, L., et al. (1991) *J. Exp. Med.* 173, 759-762; Reiser, H., et al. (1992) *Proc. Natl. Acad. Sci. USA.* 89, 271-275; van-Seventer, G.A., et al. (1990) *J. Immunol.* 144, 4579-4586; LaSalle, J.M., et al., (1991) *J. Immunol.* 147, 774-80; Dustin, M.I., et al., (1989) *J. Exp. Med.* 169, 503; Armitage, R.J., et al. (1992) *Nature* 357, 80-82; Liu, Y., et al. (1992) *J. Exp. Med.* 175, 437-445). One costimulatory pathway involved in T cell activation involves the molecule CD28 on the surface of T cells. This molecule can receive a costimulatory signal delivered by a ligand on B cells or other APCs. Ligands for CD28 include members of the B7 family of B lymphocyte activation antigens, such as B7-1 and/or B7-2 (Freedman, A.S. et al. (1987) *J. Immunol.* 137, 3260-3267; Freeman, G.J. et al. (1989) *J. Immunol.* 143, 2714-

2722; Freeman, G.J. et al. (1991) *J. Exp. Med.* 174, 625-631; Freeman, G.J. et al. (1993) *Science* 262, 909-911; Azuma, M. et al. (1993) *Nature* 366, 76-79; Freeman, G.J. et al. (1993) *J. Exp. Med.* 178, 2185-2192). B7-1 and B7-2 are also ligands for another molecule, CTLA4, present on the surface of activated T cells, although the role of CTLA4 in costimulation is unclear.

Delivery to a T cell of an antigen-specific signal with a costimulatory signal leads to T cell activation, which can include both T cell proliferation and cytokine secretion. In contrast, delivery to a T cell of an antigen-specific signal in the absence of a costimulatory signal is thought to induce a state of unresponsiveness or anergy in the T cell, thereby inducing antigen-specific tolerance in the T cell.

Interactions between T cells and B cells play a central role in immune responses. Induction of humoral immunity to thymus-dependent antigens requires "help" provided by T helper (hereafter Th) cells. While some help provided to B lymphocytes is mediated by soluble molecules released by Th cells (for instance lymphokines such as IL-4 and IL-5), activation of B cells also requires a contact-dependent interaction between B cells and Th cells (Hirohata et al., (1988) *J. Immunol.*, 140:3736-3744; Bartlett et al., (1989) *J. Immunol.*, 143:1745-1754). This indicates that B cell activation involves an obligatory interaction between cell surface molecules on B cells and Th cells. The molecule(s) on the T cell therefore mediates contact-dependent helper effector functions of the T cell. A contact-dependent interaction between molecules on B cells and T cells is further supported by the observation that isolated plasma membranes of activated T cells can provide helper functions necessary for B cell activation (Brian, (1988) *Proc. Natl. Acad. Sci. USA*, 85:564-568; Hodgkin et al., (1990) *J. Immunol.*, 145:2025-2034; Noelle et al., (1991) *J. Immunol.*, 146:1118-1124).

A molecule, CD40, has been identified on the surface of immature and mature B lymphocytes which, when crosslinked by antibodies, induces B cell proliferation (Valle et al., (1989) *Eur. J. Immunol.*, 19:1463-1467; Gordon et al., (1988) *J. Immunol.*, 140:1425-1430; Gruber et al., (1989) *J. Immunol.*, 142: 4144-4152). CD40 has been molecularly cloned and characterized (Stamenkovic et al.,(1989) *EMBO J.*, 8:1403-1410). A ligand for CD40, gp39 (also called CD40 ligand, CD40L, or CD154 (Mackey M.F. (1997) *Cancer Res* 57(13):2569-74)) has also been molecularly cloned and characterized (Armitage et al., (1992) *Nature*, 357:80-82; Lederman et al., (1992) *J.*

Exp. Med., 175:1091-1101; Hollenbaugh et al., (1992) *EMBO J.*, 11:4313-4319). The CD154 protein is expressed on activated, but not resting, $CD4^+$ Th cells (Spriggs et al., (1992) *J. Exp. Med.*, 176:1543-1550; Lane et al., (1992) *Eur. J. Immunol.*, 22:2573-2578; Roy et al., (1993) *J. Immunol.*, 151:1-14). Cells transfected with the CD154 gene and expressing the CD154 protein on their surface can trigger B cell proliferation and, together with other stimulatory signals, can induce antibody production (Armitage et al., (1992) *Nature*, 357:80-82; Hollenbaugh et al., (1992) *EMBO J.*, 11:4313-4319).

With the knowledge that T cells fail to become activated when they recognize antigen in the absence of costimulation (for a review see Mackey, M.F., et al. (1998) *J Leukocyte Biol* 63:418), methodologies for inducing tolerance centering on the suppression of costimulation have evolved. The administration of donor specific transfusion (DST) and anti-CD154 monoclonal antibodies (mAb) has been shown to induce permanent islet allograft. Once tolerance to the allograft was induced, no further modification of the immune system was required (Parker, D.C., et al (1995) *PNAS USA* 92:9560; Rossini, A.A., et al. (1996) *Cell Transplant* 5:49). When this two-element protocol was used for inducing tolerance using skin allografts, prolonged survival of murine skin allografts was demonstrated (Markees, T.G., et al. (1997) *Transplantation* 64:329). Further, this two-element protocol was successfully used to significantly prolong concordant rat to mouse islet cell xenograft survival (Markees, T.G., et al. (1996) *Transplant Proc* 28:814). The interaction of CTL-associated Ag 4 (CTLA4) with its ligands B7-1/2 may be essential in the tolerance induction process as suggested by results which show that the administration of anti-CTLA4 mAb prevents the induction of DST/anti-CD154 mediated tolerance (Markees, T.G. (1998) *J Clin Invest* 101:2446, for a review see also Rossini, A.A., et al. (1999) *Transplantation Proc* 31:629-632). Additional methodologies for inducing T cell non-responsiveness or tolerance to donor tissue, organs or cells would be of great benefit.

Summary of the Invention

The invention pertains to methods for inducing T cell non-responsiveness or tolerance to a donor tissue, organ, or cell in a recipient of the tissue, organ, or cell by administering to a transplant recipient a CD154 antagonist and a $CD8^+$ T cell depleting

agent such that non-responsiveness or tolerance of the T cell to the donor tissue, organ, or cell is induced.

The invention further pertains to a method for inducing T cell non-responsiveness or tolerance to a donor tissue, organ, or cell in a recipient of the donor tissue, organ, or cell comprising the administration to a recipient of an anti-CD154 antibody, or a fragment thereof that specifically binds CD154 and of an anti-CD8 antibody such that T cell non-responsiveness or tolerance to the donor tissue, organ, or cell is induced in the recipient.

In one embodiment, the CD154 antagonist is an anti-CD154 antibody, or a fragment thereof that specifically binds CD154. In a preferred embodiment, the anti-CD154 antibody is a monoclonal antibody. In another preferred embodiment, the monoclonal antibody is an anti-human CD154 antibody. In another preferred embodiment, the monoclonal antibody is a chimeric monoclonal antibody. In yet another preferred embodiment, the monoclonal antibody is a humanized antibody. In another preferred embodiment, the CD154 antagonist is a soluble form of a CD154 ligand. In yet another preferred embodiment, the soluble form of the CD154 ligand is a CD40 fusion protein.

In a preferred embodiment, the $CD8^+$ T cell depleting agent is an anti-CD8 antibody, or a fragment thereof that specifically binds CD8. In another preferred embodiment, the anti-CD8 antibody is a monoclonal antibody. In another preferred embodiment, the monoclonal antibody is an anti-human CD8 antibody. In yet another preferred embodiment, the monoclonal antibody is a chimeric monoclonal antibody. In another preferred embodiment, the monoclonal antibody is a humanized antibody.

In another preferred embodiment, a CD154 antagonist and a $CD8^+$ T cell depleting agent is administered to the recipient following the transplantation of the donor tissues, organs, or cells.

The invention further pertains to a method for treating a skin disorder comprising administering to a subject a CD154 antagonist, a $CD8^+$ T cell depleting agent, and allogeneic skin cells to thereby treat the skin disorder.

In another preferred embodiment, a CD154 antagonist and a $CD8^+$ T cell depleting agent is administered to the recipient following the transplantation of allogeneic skin cells.

The present invention also pertains to a method for inducing T cell non-responsiveness or tolerance to a donor tissue, organ, or cell in a recipient of a donor tissue, organ, or cell comprising administering to the recipient an anti-CD154 antibody, or a fragment thereof that specifically binds CD154 and an anti-CD8 antibody, or fragment thereof that specifically binds CD8 such that T cell non-responsiveness or tolerance is induced in the recipient.

The methods of the current invention can be used, for example, to induce T cell tolerance to transplanted tissues, organs, or cells such as pancreas, pituitary, liver, kidney, heart, lung, skin, muscle, brain tissue, nerve tissue, stomach, intestines, Adenosine Deaminase-expressing cells, and leptin-expressing cells. In one embodiment, the transplanted tissue comprises allogeneic skin graft cells. Accordingly, the invention provides a method for treating a skin disorder comprising administering to a subject in need of treatment: 1) antagonists of receptors on the surface of recipient T cells which mediate contact-dependent helper effector functions, such as CD154 antagonists and $CD8^+$ T cell depleting agents (*e.g.*, anti-CD154 and anti-CD8 antibodies); and 2) donor allogeneic skin graft cells.

Brief Description of the Drawings

Figure 1 depicts the number of cells detected in draining lymph nodes of allogeneic skin engrafted thymectomized mice after treatment with: no graft and no further treatment; graft and no treatment; graft and anti-CD154 mAb alone; graft and both anti-CD154 mAb and DST.

Figures 2A and *2B* depict the number of $CD8^+DES^+$ cells present in the spleens (2A) and lymph nodes (2B) of euthymic CBA/TCr ($H2^k$) mice, transfused with syngenic KB5 DES^+ transgenic T cells and receiving no grafts, after treatment with: no treatment; DST alone; anti-CD154 mAb alone; and both DST and anti-CD154 mAb.

Figure 3 is a representative histogram showing surface density of CD44 (left column) on $CD8^+DES^+$ spleen cells from CBA/JCr ($H2^k$) mice transfused with KB5 transgenic $CD8^+DES^+$ T cells, after treatment with: no treatment; transfusion with C57BL/6 ($H2^b$) spleen cells; anti-CD154 mAb; and both DST and anti-CD154 mAb.

Figures 4A and4B depict the cumulative survival of BALB/c skin grafts transplanted in euthymic C57BL/6 mice (4A) and C57BL/6 CD8 knock out mice (4B). In Figure 4A, the mice were treated with: anti-CD8 mAb alone; anti-CD154 mAb alone; both anti-CD8 mAb and anti-CD154 mAb; and both DST and anti-CD154 mAb. In Figure 4B, the mice were treated with: no treatment; anti-CD154 mAb at a dose of 0.25 mg/mouse; and anti-CD154 mAb at a dose of 0.5 mg/mouse.

Figures 5A and *5B* depict the number of $CD8^+DES^+$ cells present in the spleen (5A) and lymph node cells (5B) of euthymic CBA/JCr mice transfused with $CD8^+DES^+$ transgenic T cells, after treatment with: no treatment; DST and anti-CD154 mAb; and DST, anti-CD154 mAb and anti-CTLA4 mAb.

Figures 6A-6C are representative histograms depicting surface expression of CD8 (horizontal access) and the anti-H2-K^b specific TcR recognized by the anti-clonotypic mAb DES (vertical access) on lymph node cells obtained from three of the mice treated as described in Figures 5A and 5B. In particular, Figure 6A shows untreated controls, Figure 6B shows treatment with DST and anti-CD154 mAb and Figure 6C shows treatment with DST, anti-CD154 mAb and anti-CTLA4 mAb.

Detailed Description of the Invention

This invention features methods for inducing T cell non-responsiveness or tolerance *in vivo* to a donor tissue, organ, or cell in a transplant recipient. The methods involve administering to the recipient an antagonist of a receptor on the surface of the T cell which inhibits interaction of a ligand with the receptor, for example, a CD154 antagonist and a $CD8^+$ T cell depleting agent. As used herein the term "recipient" refers to a subject into whom a tissue, organ, or cell is to be transplanted, is being transplanted or has been transplanted.

According to the methods of the invention, antagonists of a molecule on T cells which mediate contact dependent helper effector functions are administered to the recipient as part of the tolerization regimen. As defined herein, a molecule or receptor which mediates contact dependent helper effector functions is one which is expressed on a Th cell and interacts with a ligand on an effector cell (*e.g.*, a B cell), wherein the interaction of the molecule with it's ligand is necessary for generation of an effector cell response (*e.g.*, B cell activation). In addition to being involved in effector cell

responses, it has now been found that such a molecule or receptor is involved in the response of the T cell to antigen. Further, it has been found that cell responsiveness can be modulated by costimulation of CD154. Preferably, the molecule on a T cell which mediates contact-dependent helper effector function is CD154. Accordingly, in preferred embodiments, the methods of the invention involve administering to a transplant recipient a CD154 antagonist. Activation of recipient T cells involves an interaction between CD154 on recipient T cells and CD154 ligands. By inhibiting these interactions with CD154 antagonists, the T cells of the recipient are not activated by the antigens expressed by allogeneic or xenogeneic cells but rather become non-responsive or tolerized to the allogeneic or xenogeneic tissue, organ or cell antigens. Induction of T cell non-responsiveness or tolerance to allogeneic or xenogeneic cell antigens in the recipient thus enables successful transplantation of the allogeneic or xenogeneic tissue, organ, or cell without immune-mediated rejection of the donor graft.

As defined herein, an "allogeneic" tissue, organ, or cell is obtained from a different individual of the same species as the recipient and expresses "alloantigens", which differ from antigens expressed by cells of the recipient. A "xenogeneic" tissue, organ, or cell is obtained from a different species than the recipient and expresses "xenoantigens", which differ from antigens expressed by cells of the recipient. As used herein, the term "donor antigens" includes antigens expressed by the donor tissue, organ, or cell to be transplanted into the recipient. The donor antigens may be alloantigens or xenoantigens, depending upon the source of the graft.

A $CD8^+$ T cell depleting agent is also administered to the recipient as part of the tolerization regimen. As defined herein, a $CD8^+$ T cell depleting agent is one which interacts with and depletes $CD8^+$ T cells from a population of T cells. It has now been found that the depletion of $CD8^+$ T cells with the concurrent inhibition of the interaction of CD154 presented on donor tissues, organs, or cells and effector cells, mediates the induction of non-responsiveness or tolerance *in vivo* to a donor tissue, organ, or cell.

Various aspects of the invention are described in further detail in the following subsections.

I. CD154 Antagonists and CD8$^+$T Cell Depleting Agents

According to the methods of the invention, a CD154 antagonist is administered to a recipient to interfere with the interaction of CD154 on recipient T cells with CD154 ligands on an allogeneic or xenogeneic cell, such as a B cell. CD154 antagonists are defined as molecules which interfere with this interaction. The CD154 antagonist can be an antibody directed against CD154 (*e.g.*, a monoclonal antibody against CD154), a fragment or derivative of an antibody directed against CD154 (*e.g.*, Fab or F(ab')$_2$ fragments, chimeric antibodies or humanized antibodies), soluble forms of a CD154 ligand (*e.g.*, soluble CD40), soluble forms of a fusion protein of a CD154 ligand (*e.g.*, soluble CD40Ig), or pharmaceutical agents which disrupt or interfere with the CD154-CD40 interaction.

According to the methods of the invention, a CD8$^+$ T cell depleting agent is administered to a recipient to deplete CD8$^+$ cells from a T cell population. CD8$^+$ T cell depleting agents are defined as molecules which mediate the depletion of CD8$^+$ T cells . The CD8$^+$ T cell depleting agent can be an antibody directed against CD8 (*e.g.*, a monoclonal antibody against CD8), a fragment or derivative of an antibody directed against CD8 (*e.g.*, Fab or F(ab)'$_2$ fragments, chimeric antibodies or humanized antibodies), and any other agent which depletes CD8$^+$ T cells such as immunotoxins.

A. Antibodies

A mammal (*e.g.*, a mouse, hamster, or rabbit), can be immunized with an immunogenic form of CD154 protein or CD8 protein or protein fragment (*e.g.*, peptide fragment) which elicits an antibody response in the mammal. A cell which expresses CD154 or CD8 on its surface can also be used as the immunogen. Alternative immunogens include purified CD154 protein, purified CD8 protein or protein fragments thereof. CD154 and CD8 can be purified from a CD154-expressing cell and CD8-expressing cell, respectively, by standard purification techniques. Additionally, CD154 cDNA (Armitage et al., *Nature*, 357:80-82 (1992); Lederman et al., *J. Exp. Med.*, 175:1091-1101 (1992); Hollenbaugh et al., *EMBO J.*, 11:4313-4319 (1992)) and/or CD8 cDNA (Nakayama, K., et al. (1989) *Immunogenetics* 30 (5):393-397 (α-chain); Shiue, L., et al. (1988) *J. Exp. Med.* 168:1993-2005 (β-chain)) can be expressed in a host cell, *e.g.*, bacteria or a mammalian cell line, and CD154 and CD8 protein purified from cell

cultures by standard techniques. Alternatively, CD154 peptides and CD8 peptides can be synthesized based upon the amino acid sequence of CD154 (disclosed in Armitage et al., *Nature*, 357:80-82 (1992); Lederman et al., *J. Exp. Med.*, 175:1091-1101 (1992); Hollenbaugh et al., *EMBO J.*, 11:4313-4319 (1992)) and the amino acid sequence of CD8 (disclosed in Nakayama, K., et al. (1989) *Immunogenetics* 30 (5):393-397 (α-chain); Shiue, L., et al. (1988) *J. Exp. Med.* 168:1993-2005 (β-chain)), using known techniques (*e.g.* F-moc or T-boc chemical synthesis). Techniques for conferring immunogenicity on a protein include conjugation to carriers or other techniques well known in the art. For example, the protein can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay can be used with the immunogen as antigen to assess the levels of antibodies.

Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera. To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art. For example, the hybridoma technique originally developed by Kohler and Milstein (*Nature* (1975) 256:495-497) as well as other techniques such as the human B-cell hybridoma technique (Kozbar et al., *Immunol. Today* (1983) 4:72), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al. *Monoclonal Antibodies in Cancer Therapy* (1985) (Allen R. Bliss, Inc., pages 77-96), and screening of combinatorial antibody libraries (Huse et al., *Science* (1989) 246:1275), may be employed. Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the protein or peptide and monoclonal antibodies isolated.

The term "CD154 antibody" as used herein is also intended to include fragments thereof which are specifically reactive with a CD154 protein or peptide thereof or CD154 fusion protein. The term "CD8 antibody" as used herein is also intended to include fragments thereof which are specifically reactive with a CD8 protein or peptide thereof or CD8 fusion protein. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, $F(ab')_2$ fragments can be generated by treating

antibody with pepsin. The resulting $F(ab')_2$ fragment can be treated to reduce disulfide bridges to produce Fab' fragments. The antibody of the present invention is further intended to include bispecific and chimeric molecules having an anti-CD154 or anti-CD8 portion.

When antibodies produced in non-human subjects are used therapeutically in humans, they are recognized to varying degrees as foreign and an immune response may be generated in the patient. One approach for minimizing or eliminating this problem, which is preferable to general immunosuppression, is to produce chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. A variety of approaches for making chimeric antibodies have been described and can be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes CD154 or CD8. See, for example, Morrison et al., *Proc. Natl. Acad. Sci. U.S.A.* 81:6851 (1985); Takeda et al., *Nature* 314:452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., European Patent Publication EP171496; European Patent Publication 0173494, United Kingdom Patent GB 2177096B. It is expected that such chimeric antibodies would be less immunogenic in a human subject than the corresponding non-chimeric antibody.

For human therapeutic purposes the monoclonal or chimeric antibodies specifically reactive with a CD154 protein or peptide or a CD8 protein or peptide, can be further humanized by producing human variable region chimeras, in which parts of the variable regions, especially the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. Such altered immunoglobulin molecules may be made by any of several techniques known in the art, (*e.g.*, Teng et al., *Proc. Natl. Acad. Sci. U.S.A.*, 80:7308-7312 (1983); Kozbor et al., *Immunology Today*, 4:7279 (1983); Olsson et al., *Meth. Enzymol.*, 92:3-16 (1982)), and are preferably made according to the teachings of PCT Publication WO92/06193 or EP 0239400. Humanized antibodies can be commercially produced by, for example, Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.

Another method of generating specific antibodies, or antibody fragments, reactive against a CD154 protein or peptide or a CD8 protein or peptide, is to screen expression libraries encoding immunoglobulin genes, or portions thereof, expressed in bacteria with a CD154 protein or peptide, or CD8 protein or peptide, respectively. For example, complete Fab fragments, VH regions and FV regions can be expressed in bacteria using phage expression libraries. See, for example, Ward et al., *Nature*, 341: 544-546: (1989); Huse et al., *Science*, 246: 1275-1281 (1989); and McCafferty et al., *Nature*, 348: 552-554 (1990). Screening such libraries with, for example, a CD154 peptide can identify immunoglobin fragments reactive with CD154. Likewise, screening such libraries with, for example, a CD8 peptide can identify immunoglobin fragments reactive with CD8. Alternatively, the SCID-hu mouse (available from Genpharm) can be used to produce antibodies, or fragments thereof.

Anti-CD154 monoclonal antibodies of the invention are preferred for use in inducing antigen-specific T cell tolerance. Preferred antibodies include monoclonal antibody MR1, a hamster anti-mouse CD154 mAb produced as ascites in *scid* mice (Noelle, R.J., et al. (1992) *PNAS USA* 89:6550; Foy, T.M., et al. (1993) *J Exp Med* 178:1567).

Anti-human CD8 monoclonal antibodies of the invention are also preferred for use in inducing antigen-specific T cell tolerance. Preferred antibodies include rat monoclonal antibodies directed against mouse CD8 (hybridoma clone 2.43, available from American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209 USA; Kruisbeek, A.M. (1994) In *Current Protocols in Immunology*, Coligan, J.E., et al. Ed.s, John Wiley & Sons, New York. 4.1.1-4.1.5).

In another embodiment, an anti-human CD154 mAb for use in the methods of the invention binds an epitope recognized by the monoclonal antibody MR1. Likewise, an anti-human CD8 mAb for use in the methods of the invention binds an epitope recognized by the monoclonal antibody against CD8. The ability of an mAb to bind an epitope recognized by the aforementioned antibodies can be determined by standard cross-competition assays. For example, an antibody that binds the same epitope recognized by MR1 will compete for the binding of labeled CD154 to activated T cells, whereas an antibody that binds a different epitope than that recognized by MR1 will not compete for the binding of labeled CD154 to activated T cells.

B. Soluble Ligands

Other CD154 antagonists which can be administered to induce T cell tolerance include soluble forms of a CD154 ligand. A monovalent soluble ligand of CD154, such as soluble CD40, can bind to CD154, thereby inhibiting the interaction of CD154 with CD40 on B cells. The term "soluble" indicates that the ligand is not permanently associated with a cell membrane. A soluble CD154 ligand can be prepared by chemical synthesis, or, preferably by recombinant DNA techniques, for example by expressing only the extracellular domain (absent the transmembrane and cytoplasmic domains) of the ligand. A preferred soluble CD154 ligand is soluble CD40. Alternatively, a soluble CD154 ligand can be in the form of a fusion protein. Such a fusion protein comprises at least a portion of the CD154 ligand attached to a second molecule. For example, CD40 can be expressed as a fusion protein with immunoglobulin (i.e., a CD40Ig fusion protein). In one embodiment, a fusion protein is produced comprising amino acid residues of an extracellular domain portion of CD40 joined to amino acid residues of a sequence corresponding to the hinge, CH2 and CH3 regions of an immunoglobulin heavy chain, *e.g.* Cγ1, to form a CD40Ig fusion protein (see *e.g.*, Linsley et al. (1991) *J. Exp. Med.* 1783:721-730; Capon et al. (1989) *Nature* 337, 525-531; and Capon U.S. 5,116,964). The fusion protein can be produced by chemical synthesis, or, preferably by recombinant DNA techniques based on the cDNA of CD40 (Stamenkovic et al., *EMBO J.*, 8:1403-1410 (1989)).

Other $CD8^+$ T cell depleting agents which can be administered to induce T cell tolerance include immunotoxins. As used here, the term "immunotoxin" includes toxins or their subunits covalently coupled to another agent such as a monoclonal antibody or soluble ligand. These molecules mediate a toxic interaction between the toxin moiety and the target cell *e.g.*, those displaying an epitope recognized by the monoclonal moiety (Frankel, A.E. (1993) *Oncology (Huntingt)* 7(5):69-78).

II. Administration of CD154 antagonists and $CD8^+$ T cell depleting agents

T cell tolerance to an tissue, organ, or cell can be induced according to the invention by administration to the transplant recipient CD154 antagonists and $CD8^+$ T cell depleting agents which interact with recipient T cells via CD154 and mediate the

depletion of $CD8^+$ T cells. In a preferred embodiment, the CD154 antagonists and $CD8^+$ T cell depleting agents are administered to the recipient simultaneously or contemporaneously. In a preferred embodiment, the antagonists are administered to the recipient following transplantation of the tissue, organ, or cell into the recipient. For example, administration of the antagonists can be performed over several days (*e.g.*, beginning during transplantation until day four) following tissue, organ, or cell transplantation.

Antagonists and agents of the invention are administered to a subject in a biologically compatible form suitable for pharmaceutical administration *in vivo* to induce T cell tolerance. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the antagonists or agent to be administered in which any toxic effects are outweighed by the therapeutic effects of the compound. The term "subject" is intended to include living organisms in which an immune response can be elicited, *e.g.*, mammals. Examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof. CD154 antagonists and $CD8^+$ T cell depleting agents can be administered in any pharmacological form, optionally with a pharmaceutically acceptable carrier. Administration of a therapeutically active amount of the antagonist and agent is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result (*e.g.*, T cell tolerance). For example, a therapeutically active amount of antagonists of CD154 and $CD8^+$ T cell depleting agents may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the antagonists and agents to elicit a desired response in the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. An effective treatment regimen can include initiation of antibody administration during tissue, organ, or cell transplantation, followed by readministration of the antibodies (*e.g.*, every day) for four days after transplantation.

The active compound (*e.g.*, an antagonist or agent, such as antibodies) may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active compound may be coated in a

material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound. A preferred route of administration is by intravenous injection.

To administer antagonists of CD154 and $CD8^+$ T cell depleting agents other than by parenteral administration, it may be necessary to coat the antagonists and agents with, or co-administer the antagonists and agents with, a material to prevent their inactivation. For example, antagonists and agents can be administered to an individual in an appropriate carrier or diluent, co-administered with enzyme inhibitors or in an appropriate carrier such as liposomes. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEP) and trasylol. Liposomes include water-in-oil-in-water emulsions as well as conventional liposomes (Strejan et al., (1984) *J. Neuroimmunol* 7:27).

The active compound may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases, the composition must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol and sodium chloride in the composition.

Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating active compound (*e.g.*, antagonists of CD154 and $CD8^+$ T cell depleting agents) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient (*e.g.*, antagonist, depleting agent) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

When the active compound is suitably protected, as described above, the protein may be orally administered, for example, with an inert diluent or an assimilable edible carrier. As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with thc active compound, use thereof in the therapeutic compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

Subsequent to or concurrent with the tolerization regimen described herein, a donor tissue, organ, or cell is transplanted into a transplant recipient by conventional techniques.

III. Uses of the Methods of the Invention

The methods of the invention are applicable to a wide variety of tissue, organ, and cell transplant situations. The methods can be used to induce T cell tolerance in a recipient of a graft of a tissue, organ, or cell such as pancreas, pituitary, liver, kidney, heart, lung, skin, muscle, brain tissue, nerve tissue, stomach, intestines, Adenosine Deaminase-expressing cells, and leptin-expressing cells. Thus, the methods of the invention can be applied in treatments of diseases or conditions which entail tissue, organ, or cell transplantation (*e.g.*, liver transplantation to treat hypercholesterolemia, transplantation of muscle cells to treat muscular dystrophy, transplantation of neuronal tissue to treat Huntington's disease or Parkinson's disease, transplantation of tissues expressing thyroid hormone for the treatment of thyroid disease, transplantation of tissues expressing insulin for the treatment of diabetes, transplantation of allogeneic skin grafts in burn patients, transplantation of leptin-expressing tissues for the treatment of weight regulatory disorders, etc.) and for the alleviation of genetic defects, for example, the transplantation of Adenosine Deaminase-expressing cells to treat Adenosine Deaminase deficient severe combined immunodeficiency (ADA). In a preferred embodiment, the transplanted tissue comprises allogeneic skin graft cells. Accordingly, the invention encompasses a method for treating a skin disorder by allogeneic skin graft transplantation. The method comprises administering to a subject in need of treatment: 1) antagonists of molecules expressed on recipient T cells which mediate contact-dependent helper effector function, such as CD154 antagonists, and $CD8^+$ T cell depleting agents (*e.g.*, anti-CD154 and anti-CD8 antibodies) and 2) donor allogeneic skin graft cells. Preferably, the antagonists and agents are administered to the recipient following administration of the allogeneic skin graft.

The invention is further illustrated by the following example which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are hereby incorporated by reference.

EXAMPLES

The following materials and methods were used in the Examples.

Pretreatment of Graft Recipients

CBA/JCr ($H2^k$), C57BL/6 ($H2^b$) and BALB/c ($H\text{-}2^d$) mice were obtained from the National Cancer Institute (Frederick, MD). C57BL/6 mice in which the CD8 lymphocyte surface antigen gene was disrupted by homologous recombination were obtained from The Jackson Laboratory (Bar Harbor, ME). To investigate the fate of specific alloreactive T cells, a KB5 TcR transgenic mouse was established in the animal colony, which has specificity to native $H2^b$ alloantigen (Tafuri, A., et al, (1995) *Science* 270:630; Kearney, E.R., et al, (1994) *Immunity* 1:327). The TcR transgene is expressed by $CD8^+$ cells in CBA ($H2^k$) mice and has specificity for $H2\text{-}K^b$.

All animals were certified to be free of Sendai virus, pneumonia virus of mice, murine hepatitis virus, minute virus of mice, ectromelia, LDH elevating virus, GD7 virus, Reo-3 virus, mouse adenovirus, lymphocytic choriomeningitis virus, polyoma, *Mycoplasma pulmonis*, and *encephalitozoon cuniculi*. All animals were housed in microisolator cages, given *ad libitum* access to autoclaved food, and maintained in accordance with recommendations in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996) and the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School.

PCR Typing of KB5 TcR Transgenic Mice

KB5 TcR transgenic mice were typed by PCR using DNA from ear punches (Chen, S.Z., et al, (1990) *Biotechniques* 8:32). The PCR primers specific for the transgenic alpha chain were designed using Oligo primer analysis software (National Biosciences) and the published sequence (Hue, I.J., et al, (1990) J. *Immunol* 144:4410). The forward primer, 5'-GCAGCAGGTGAGACAAA-3' (specific for the V region), and the reverse primer, 5'-ATACCGTGGTTCCTGTTC-3' (specific for the J segment), produce a 323 bp product from the rearranged gene. PCR was performed in the presence of 2 mM $MgCl_2$ at an annealing temperature of 55°C.

Transplantation procedure

Male C57BL/6 or CBA/JCr recipient mice 6-8 weeks of age were tolerized and transplanted with skin allografts using previously published techniques (Markees, T.G., et al, (1998) *J. Clin. Invest.* 101:2446; Markees, T.G., et al, (1997) *Transplantation* 64:329). Briefly, 10^7 C57BL/6 or BALB/c splenocytes from female retired breeder donors were injected intravenously in a volume of 0.5 ml at various times prior to transplantation into CBA/JCr or C57BL/6 skin graft recipients, respectively. The MR1 hamster anti-mouse CD154 mAb was produced as ascites in scid mice (Noelle, R.J., et al, (1992) *Proc. Natl. Acad. Sci.* 89:6550; Foy, T.M., et al, (1993) *J. Exp. Med.* 178:1567). Antibody concentration in ascites was determined by ELISA. Anti-CD154 mAb was administered intraperitoneally at a dose of 0.25 mg or 0.5 mg per mouse on varying schedules.

C57BL/6 or BALB/c skin grafts 1-2 cm in diameter were transplanted onto the dorsal flanks of recipient CBA/JCr or C57BL/6 mice, respectively. Graft rejection was defined as the first day on which the entire graft was necrotic (Markees, T.G., et al, (1998) *J. Clin. Invest.* 101:2446; Markees, T.G., et al, (1997) *Transplantation* 64:329). In certain experiments, recipients were thymectomized as previously described (Reeves, J.P., et al, (1994) *In Current Protocols in Immunology* 1.10.1-1.10.11).

Anti-CD8 and Anti-CTLA4 mAb Administration

Hybridoma cells secreting a depleting rat mAb directed against mouse CD8 (clone 2.43) were obtained from the American Type Culture Collection, Manassas, VA MD (Kruisbeek, A.M., (1994) *In vivo* 4.1.1-4.1.5). To deplete $CD8^+$ cells, mice were given 0.5 mg of mAb intraperitoneally daily for 3 days. This protocol was documented by flow microfluorometry to deplete >95% of $CD8^+$ T cells 48 hr after the last dose of mAb. A hybridoma cell line secreting hamster anti-mouse CTLA4 mAb (clone 9H10) was used. Anti-CTLA4 mAb was injected intraperitoneally at a dose of 0.075 mg per mouse daily for 3 days. Both antibodies were produced as ascites in *scid* mice. For the anti-CD8 mAb, rat immunoglobulin concentration was measured by radial immunodiffusion (The Binding Site, San Diego, CA); for the anti-CTLA4 mAb, hamster immunoglobulin concentration was measured by ELISA.

Analysis of Cell Number

In preliminary experiments, the lymph nodes that drain skin grafts placed in the standard flank location were identified. To do so, Evans Blue dye (0.01 ml of 2.5% wt/vol) was injected directly either into the bed prepared for skin grafts or directly into successful skin grafts on mice that had been treated with DST and anti-CD154 mAb. In both cases, dye was subsequently observed only in the axillary and lateral axillary nodes (Cook, M.J., (1983) *Anatomy* 101-120). For cell counts, lymph nodes or intact spleens were dissected free of other tissues and extruded through a cell sieve. The total number of viable mononuclear cells present was determined by the method of Trypan blue exclusion using a hemocytometer. Cell viability was >95% in all cases.

Flow Microfluorometry

Phycoerythrin (PE) conjugated anti-mouse mAbs directed against CD4 (clone GK1.5) and the activation markers CD44 (clone IM7) and CD62L (Mel-14) were obtained from Pharmingen (San Diego, CA). Cy-Chrome™-conjugated anti-mouse CD8α mAb (clone 53-6.7) was also obtained from Pharmingen. A hybridoma cell line secreting the clonotypic DES antibody (Tafuri, A., et al, (1995) *Science* 270:630) was used. Isotype control mAbs including the PE conjugated rat IgG2a kappa (clone R35-95) for CD44 and CD4, PE-conjugated rat IgG2b kappa (clone A95-1) for CD62L, rat Cy-Chrome™-conjugated IgG2a kappa (clone R35-95) for CD8, and mouse IgG2a kappa anti-TNP (clone G155-178) for DES were obtained from Pharmingen.

Two and three-color flow cytometric analyses were performed as previously described (Zadeh, et al, (1996) *Autoimmunity* 24:35). Briefly, 1 x 10^6 viable lymph node or spleen T cells were reacted with a mixture of conjugated mAbs for 20 min. at 4°C. Cells were then washed and fixed with 2% paraformaldehyde. KB5 cells were incubated in the presence of anti-DES antibody, washed, and then reacted with FITC-conjugated anti-mouse IgG2a (Pharmingen). Cells were washed and fixed with 2% paraformaldehyde. Labeled cells were analyzed using a FACScan® instrument (Becton Dickinson, Sunnyvale, CA). Lymphoid cells were gated according their light-scattering properties. Levels of background fluorescence were subtracted.

In some experiments, the forward light scatter characteristics of lymphoid cells were quantified and used as an index of cell size and, by extension, of cell activation. In these experiments, the forward scatter parameter (in arbitrary units) obtained from all untreated controls analyzed at the same time was averaged. For analysis, the ratio of each control and experimental value (obtained during the same run) to that mean control value was calculated. This procedure was adopted to account for day to day variability in machine settings, which caused variation in the forward scatter parameter associated with control cells. In all instances, a minimum of 50,000 events was acquired for each analysis.

T Cell Transgenic Chimeras

To examine the fate of alloreactive $CD8^+$ T cells under conditions that are "normal," the adoptive transfer chimera system originally described by Jenkins and colleagues (Kearney, E.R., et al, (1994) *Immunity* 1:327; Kearney, E.R., et al, (1995) *J.Immunol* 155:1032) was used. In this system, small numbers of transgenic T cells are injected into syngeneic non-transgenic hosts to permit the engraftment of low levels of tracer transgenic cells in a host cellular microenvironment that is essentially unaltered by transgene expression. Transgenic T cells were enriched from spleen and lymph nodes of CBA-derived transgenic KB5 mice ($H2^k$). These transgenic T cells express an anti-$H2\text{-}K^b$ specific TcR that is recognized by the anti-clonotypic mAb DES (Tafuri, A., (1995) *Science* 270:630). Prior to their transfusion into adoptive recipients, spleen and lymph node cells from KB5 mice were enriched for the $CD8^+DES^+$ population using a CD8 Cellect™ column purification kit (Biotex Laboratories, Edmonton , Canada). Briefly, spleens and lymph nodes were removed and gently extruded through nylon sieves in cold PBS containing 2% FBS. Erythrocytes were lysed with hypotonic NH_4Cl and the samples reacted with rat anti-mouse CD4 mAb. The cell suspension was then loaded onto the column (which is provided with bound goat anti-mouse Ig) and eluted with PBS-2% FBS. Cells not retained by the column were analyzed by flow microfluorometry and found to contain <0.1% $CD4^+$ cells and 60-80% $CD8^+DES^+$ cells. CBA/JCr ($H2^k$) skin allograft recipients and controls were transfused with 1-3 x 10^6 purified transgenic T cells. The number of $CD8^+DES^+$ cells present in adoptive recipients at various times after transfusion was analyzed by flow microfluorometry.

Statistics

In the Figures, average duration of graft survival is presented as the median. Graft survival among groups was compared using the method of Kaplan and Meier (Kaplan, E.L., et al, (1958) *J. Am. Statist. Assn.* 53-457). The equality of allograft survival distributions for animals in different treatment groups was tested using the log rank statistic (Kaplan, E.L., et al, (1958) *J. Am. Statist.Assn.* 53:457). P values <0.05 were considered statistically significant. Comparisons of three or more arithmetic means used one-way analyses of variance (Nie, N.H., (1975) *Statistical Package for Social Sciences* 1-675) and either the least significant difference procedure or Bonferroni adjusted unpaired t-tests (Glantz, S.A., (1981) *Primer of Biostatistics* 352) for *a posteriori* contrasts.

Example 1: Induction of tolerance to skin grafts by treatment of recipients with DST and anti-CD154 antibodies

To determine the induction of tolerance to skin grafts by treatment of recipients with DST and anti-CD154 antibodies, the total number of cells present in the axillary and lateral axillary lymph nodes in five groups of thymectomized mice were measured. The control group was untreated; the remaining groups were comprised of skin graft recipients that were otherwise untreated, or treated with DST and anti-CD154 mAb alone or in combination. The total number of cells present in untreated control lymph nodes ranged between 2.7-3.1 x 10^6 and remained constant over time (Table 1). In contrast, the number of cells present in the draining axillary nodes of otherwise untreated skin graft recipients was more than 3 times greater on day 7 (by which time grafts had all rejected). It was 8 times greater on day 9 ($p<0.001$ vs. untreated, ungrafted controls). Lymph node cell numbers in skin graft recipients that had received either anti-CD154 mAb or DST as monotherapy were also much higher than in ungrafted controls on days 9 ($p<0.025$) and 13 ($p<0.001$) after grafting (Table 1). As expected (Markees, T.G., (1997) *Transplantation* 64:329), mice in these groups uniformly rejected their grafts by day 13. In contrast, the number of cells present in draining axillary nodes of graft recipients treated with both anti-CD154 mAb and DST was statistically similar to the number of cells present in the ungrafted, untreated controls at each time point. Mice in this last group also had healthy grafts on day 13, the final day of observation. To

exclude any influence of thymectomy on these results, comparable data were also obtained in a similar experiment conducted using five groups of non-transgenic, euthymic mice treated in the same way (data not shown).

TABLE 1

CELL NUMBERS IN LYMPH NODES DRAINING SKIN ALLOGRAFTS

	Cell Number (x 10^6)				
Days After Skin Grafting	*Ungrafted Control*	*Skin Graft Only*	*Skin Graft + DST*	*Skin Graft + Anti-CD154 mAb*	*Skin Graft + Anti-CD154 mAb + DST*
7	2.8 ± 1.0 (3)	9.8 ± 1.5 (3)[a]	4.8 ± 1.3 (3)	2.6 ± 0.7 (3)	3.9 ± 0.8 (3)
9	2.7 ± 0.5 (6)[b]	26.2 ± 11.7 (6)	14.8 ± 4.1 (6)	11.1 ± 2.0 (6)	5.7 ± 2.1 (6)
13	3.1 ± 0.9 (5)[c]	11.9 ± 2.6 (7)	N.D.	11.8 ± 5.2 (8)	5.3 ± 2.5 (8)

Each data point represents the mean ± 1 s.d. of 3-8 animals (N). N.D.: not determined. a: $p<0.001$ *vs.* all other day 7 groups. b: $p<0.025$ *vs.* graft only, *vs.* graft + DST and *vs.* graft + anti-CD154 mAb groups, but p=N.S. *vs.* graft plus combined therapy. c: $p<0.001$ *vs.* graft only and *vs.* graft + anti-CD154 mAb groups, but p=N.S. *vs.* graft plus combined therapy. No other paired comparisons within rows were statistically significant.

Example 2: Determination of the fate of alloreactive cells in graft-draining lymph nodes

To determine the fate of alloreactive cells in graft-draining lymph nodes, four groups of euthymic CBA/JCr ($H2^k$) mice were transfused with syngeneic KB5 DES^+ transgenic T cells (specific for H-$2K^b$). Four groups of euthymic CBA/JCr ($H2^k$) mice were transfused with a tracer population of syngeneic KB5 DES^+ transgenic T cells (specific for H-$2K^b$). One group received no further treatment. The other three groups received a C57BL/6 ($H2^b$) skin graft plus either no additional treatment, anti-CD154 mAb alone, or both anti-CD154 mAb and DST. The interval between the first injection of anti-CD154 mAb and transplantation on day 0 was ~84 hours. The number of $CD8^+DES^+$ cells present in the draining lymph nodes in all groups was measured on day +7 relative to transplantation (Figure 1). In Figure 1, each data point represents the mean $\pm$1 s.d. of 3-6 animals as indicated by the numbers in parenthesis.

The number of $CD8^+DES^+$ T cells in the draining lymph nodes of ungrafted, untreated euthymic controls was $4.1\pm1.7 \times 10^3$, representing ~0.5-0.8% of the total lymph node cell population. The number of $CD8^+DES^+$ T cells in the axillary lymph nodes of otherwise untreated graft recipients ($52.7\pm17.9 \times 10^3$) was much greater than in the ungrafted controls (Figure 1, $p<0.001$). The number of $CD8^+DES^+$ T cells in graft recipients treated with anti-CD154 mAb monotherapy ($27.8\pm16.5 \times 10^3$, $p<0.001$) was also significantly greater than in the ungrafted controls, but less than in the untreated graft recipients ($p<0.05$). In striking contrast, the number of $CD8^+DES^+$ T cells in the draining lymph nodes of skin allograft recipients treated with both DST and anti-CD154 mAb ($0.9 \pm 0.4 \times 10^3$, Figure 1) was not only less than that in the nodes of the other graft recipient groups, but also significantly *less* than in the ungrafted controls ($p<0.025$, Figure 1).

Example 3: Determination of $CD8^+$ alloreactive transgenic T cell depletion

To determine whether $CD8^+$ allogeneic transgenic T cells were depleted, four groups of euthymic CBA/JCr ($H2^k$) mice were transfused with syngeneic KB5 DES^+ transgenic T cells. None of the animals received grafts, but they did receive either no treatment, DST alone, anti-CD154 mAb alone, or both anti-CD154 mAb and DST. Cell

number was measured 84 hours later, the time point at which the grafts had been transplanted in Example 2. The number of $CD8^+DES^+$ cells present in the spleens of mice treated with DST and anti-CD154 mAb was statistically significantly less than that in any other treatment group ($p<0.03$, Figure 2A). The number of $CD8^+DES^+$ cells present in a pooled preparation of axillary and inguinal lymph nodes was also significantly lower in mice treated with DST and anti-CD154 mAb than that in any other treatment group ($p<0.002$, Figure 2B). The number of $CD8^+DES^+$ cells present in the lymph nodes of mice treated with DST alone was significantly higher than in any other treatment group ($p<0.005$, Figure 2B). For mice treated with both DST and anti-CD154 mAb, the number of $CD8^+DES^+$ cells present in both spleen and lymph node was <10% of the number present in untreated controls.

Example 4: Determination of $CD8^+$ alloreactive transgenic T cell activation before depletion

To determine the status of activation in $CD8^+$ alloreactive transgenic T cells before depletion, four groups of euthymic CBA/JCr ($H2^k$) mice were transfused with a tracer population of syngeneic KB5 DES^+ transgenic T cells. None of the animals received grafts. The groups received either no further treatment, DST alone, anti-CD154 mAb alone, or both anti-CD154 mAb plus DST. Spleens and lymph nodes were recovered 48 hours later and counted. KB5 $CD8^+DES^+$ cells were analyzed by flow microfluorometry for forward and side light scatter (as an index of size) and for surface expression of CD44 and CD62L, markers indicative of an activated state (Croft, M. et al, (1997) *CRC Critc. Rev. Immunol* 17:89).

It was found that the number of $CD8^+DES^+$ spleen cells and lymph node cells at the 48 hour time point was statistically similar in all four groups (Table 2). However, for each of the three parameters used as an index of activation, it was observed that the results for both of the groups that had been treated with DST were significantly different from those obtained in either the untreated group or mAb monotherapy group (Table 2). In all cases, both spleen and lymph node $CD8^+DES^+$ cells from the groups treated with DST (alone or with anti-CD154 mAb) were larger and evidenced upregulation of CD44 and downregulation of CD62L. There were no statistically significant differences between the untreated controls and the anti-CD154 mAb monotherapy group. In

addition, there were no statistically significant differences between the DST monotherapy and DST plus anti-CD154 mAb groups for any of the three parameters, indicating that the antibody had not prevented T cell activation. Representative histograms illustrating the expression of CD44 and CD62L on $CD8^+DES^+$ cells for each of the four experimental groups are shown in Figure 3. In these histograms, cell number (vertical axis) is plotted against fluorescence intensity (horizontal axis). Background isotype control values are shown in uppermost panels. Percentages of $CD44^+$ and $CD26L^{low}$ cells are given in Table 2.

$CD8^+DES^+$ cells from identical groups of animals obtained 84 hr after treatment were examined. $CD8^+DES^+$ cells from mice treated with either DST or anti-CD154 mAb as monotherapy also exhibited activated and non-activated phenotypes, respectively (data not shown). The phenotype of $CD8^+DES^+$ cells from mice treated with both DST and anti-CD154 mAb could not be determined because, consistent with the results in Figure 2, almost no $CD8^+DES^+$ cells were present.

TABLE 2

LIGHT SCATTTER PROPERTIES AND PHENOTYPE OF $CD8^{+}DES^{+}$ SPLENOCYTES AND LYMPH NODE CELLS

	Control (N=5)	*Anti-CD154 mAb Alone (N=6)*	*DST Alone (N=6)*	*Anti-CD154 mAb + DST (N=6)*
Spleen Cells				
Cell Number (x 10^5)[a]	2.3±0.7	2.4±0.6	2.0±0.6	1.6±0.7
Light Scatter Index	1.00±0.00[b]	1.03±0.04[c]	1.20±0.10[d]	1.22±0.92
CD44hi (%)	24.2±6.9[b]	26.7±4.5[c]	90.7±2.3[d]	91.5±2.9
CD62lo (%)	9.8±4.8[b]	14.7±8.9[c]	38.7±10.9[d]	43.2±13.0
Lymph Node Cells				
Cell Number (x 10^3)[a]	33.6±6.5	30.1±9.3	20.6±11.4	23.6±13.3
Light Scatter Index	1.00±0.01[b]	1.00±0.01[c]	1.22±0.09[d]	1.28±0.09
CD44hi (%)	17.0±5.5[b]	17.2±7.0[c]	86.7±3.7[d]	85.3±6.1
CD62lo (%)	15.6±7.8[b]	15.3±8.2[c]	34.5±9.4[d]	36.7±10.8

a: Spleen and lymph node cell numbers from mice in each of the four groups were statistically similar. b: Statistically similar to mAb alone group, and $p<0.05$ *vs.* both DST and DST plus anti-CD154 mAb groups. c: $p<0.05$ *vs.* DST and DST plus anti-CD154 mAb groups. d: Statistically similar to DST plus anti-CD154 mAb group. No other paired comparisons within rows were statistically significant.

Example 5: Determination of tolerance induction by co-administration of anti-CD8 and anti-CD154 antibodies

To determine the induction of tolerance by co-administration of anti-CD8 and anti-CD154 mAbs, a cohort of euthymic C57BL/6 mice was randomized to receive either anti-CD8 mAb alone, anti-CD154 mAb alone, combined therapy with both mAbs, or combined therapy with DST and anti-CD154 mAb. All mice also received a BALB/c skin graft. As shown in Figure 4A, a course of anti-CD154 mAb plus a depleting anti-CD8 mAb prolonged skin allograft survival (MST = 76 days) that was statistically similar to that achieved with DST and anti-CD154 mAb (MST = 95 days). Monotherapy with either anti-CD154 mAb (MST = 12 days) or anti-CD8 mAb alone (MST = 10 days) did not prolong skin allograft survival.

Example 6: Anti-CD154 mAb monotherapy prolongs skin allograft survival in CD8 knockout mice

The survival of BALB/c skin allografts on C57BL/6 CD8 knockout mice was analyzed. Three groups of graft recipient mice were randomized to receive either no additional treatment or anti-CD154 mAb monotherapy on one of two dosing schedules. Median graft survival in untreated mice was 14 d. At the lower dose of mAb, median graft survival was prolonged to 23 d ($p<0.03$), and at the higher dose it was prolonged to 57 d ($p<0.01$, Figure 4B).

Example 7: Determination of effect of anti-CTLA4 antibodies on T cell activation

To determine the effect of anti-CTLA4 mAbs on T cell activation, a cohort of euthymic CBA/JCr mice was injected with a tracer population of $CD8^+DES^+$ transgenic T cells and then randomized 2 days later into three groups. Group 1 received no further treatment. Group 2 received standard treatment with DST and anti-CD154 mAb. Group 3 received DST and anti-CD154 mAb plus three daily injections of anti-CDLA4 mAb, beginning on the day of DST.

Consistent with the results shown in Figure 2, the number of $CD8^+DES^+$ cells present in the spleens of mice treated with DST and anti-CD154 mAb was statistically significantly less than in untreated controls ($p<0.005$, Figure 5A). In contrast, the number of $CD8^+DES^+$ cells present in mice treated with DST, anti-CD154 mAb, and

anti-CTLA4 mAb not only failed to decline, but actually expanded by an order of magnitude ($p<0.003$ *vs.* other groups, Figure 5A). Similarly, the number of $CD8^+DES^+$ cells present in a pooled preparation of axillary and inguinal lymph nodes from mice treated with DST, anti-CD154 mAb was statistically significantly less than in untreated controls ($p<0.005$, Figure 5B). Again, the addition of anti-CTLA4 mAb to DST and anti-CD154 mAb was associated with a dramatic expansion of $CD8^+DES^+$ cells ($p<0.003$ *vs.* other groups, Figure 5B). Representative histograms illustrating the deletion of the $CD8^+DES^+$ population in the presence of DST and anti-CD154 mAb, and its expansion in the presence of anti-CTLA4 mAb are shown in Figure 6. The arrows indicate the population of $CD8^+$ DES^+ cells in each of the three treatment conditions.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims. The contents of all references and published patent applications cited throughout this application are hereby incorporated by reference.

CLAIMS

1. A method for inducing T cell non-responsiveness to a donor tissue, organ or cell in a recipient of the tissue, organ or cell comprising administering to the recipient:

a) a CD154 antagonist; and

b) a $CD8^+$ T cell depleting agent, such that non-responsiveness of the T cell to the donor tissue, organ, or cell is induced.

2. The method of claim 1, wherein a CD154 antagonist is an anti-CD154 antibody, or a fragment thereof that specifically binds CD154.

3. The method of claim 2, wherein the anti-CD154 antibody is a monoclonal antibody.

4. The method of claim 3, wherein the monoclonal antibody is an anti-human CD154 antibody.

5. The method of claim 3, wherein the monoclonal antibody is a chimeric monoclonal antibody.

6. The method of claim 3, wherein the monoclonal antibody is a humanized monoclonal antibody.

7. The method of claim 1, wherein the CD154 antagonist is a soluble form of a CD154 ligand.

8. The method of claim 7, wherein the soluble form of a CD154 ligand is a CD40 fusion protein.

9. The method of claim 1, wherein the $CD8^+$ T cell depleting agent is an anti-CD8 antibody, or fragment thereof that specifically binds CD8.

10. The method of claim 9, wherein the anti-CD8 antibody is a monoclonal antibody.

11. The method of claim 10, wherein the monoclonal antibody is an anti-human CD8 antibody.

12. The method of claim 10, wherein the monoclonal antibody is a chimeric monoclonal antibody.

13. The method of claim 10, wherein the monoclonal antibody is a humanized monoclonal antibody.

14. The method of claim 1, wherein the CD154 antagonist and the $CD8^+$ T cell depleting agent are administered to the recipient following transplantation of the donor tissue, organ, or cell.

15. The method of claim 1, wherein the tissue, organ, or cell is selected from the group consisting of pancreas, pituitary, liver, kidney, heart, lung, skin, muscle, brain tissue, nerve tissue, stomach, intestine, Adenosine Deaminase-expressing cells, and leptin-expressing cells.

16. A method for treating a skin disorder comprising administering to a subject:

a) a CD154 antagonist;

b) a $CD8^+$ T cell depleting agent; and

c) donor allogeneic skin cells, to thereby treat the skin disorder.

17. The method of claim 16, wherein a CD154 antagonist is an anti-CD154 antibody, or a fragment thereof that specifically binds CD154.

18. The method of claim 17, wherein the anti-CD154 antibody is a monoclonal antibody.

19. The method of claim 18, wherein the monoclonal antibody is an anti-human CD154 antibody.

20. The method of claim 18, wherein the monoclonal antibody is a chimeric monoclonal antibody.

21. The method of claim 18, wherein the monoclonal antibody is a humanized monoclonal antibody.

22. The method of claim 16, wherein the CD154 antagonist is a soluble form of a CD154 ligand.

23. The method of claim 22, wherein the soluble form of a CD154 ligand is a CD40 fusion protein.

24. The method of claim 16, wherein a $CD8^+$ T cell depleting agent is an anti-CD8 antibody, or a fragment thereof that specifically binds CD8.

25. The method of claim 24, wherein the anti-CD8 antibody is a monoclonal antibody.

26. The method of claim 25, wherein the monoclonal antibody is an anti-human CD8 antibody.

27. The method of claim 25, wherein the monoclonal antibody is a chimeric monoclonal antibody.

28. The method of claim 25, wherein the monoclonal antibody is a humanized monoclonal antibody.

29. The method of claim 16, wherein the CD154 antagonist and the $CD8^+$ T cell depleting agent are administered to the recipient following transplantation of the allogeneic skin cells.

30. A method for inducing T cell non-responsiveness to a donor tissue, organ, or cell in a recipient of the donor tissue, organ, or cell comprising administering to the recipient an anti-CD154 antibody, or a fragment thereof that specifically binds CD154 and an anti-CD8 antibody such that T cell non-responsiveness to the donor tissue, organ, or cell is induced in the recipient.

31. The method of claim 30, wherein the anti-CD154 antibody is a monoclonal antibody.

32. The method of claim 31, wherein the monoclonal antibody is a chimeric monoclonal antibody.

33. The method of claim 31, wherein the monoclonal antibody is a humanized monoclonal antibody.

34. The method of claim 30, wherein the anti-CD154 antibody is an anti-human CD154 antibody.

35. The method of claim 30, wherein the anti-CD8 antibody is a monoclonal antibody.

36. The method of claim 35, wherein the monoclonal antibody is a chimeric monoclonal antibody.

37. The method of claim 35, wherein the monoclonal antibody is a humanized monoclonal antibody.

38. The method of claim 30, wherein the anti-CD8 antibody is an anti-human CD8 antibody.

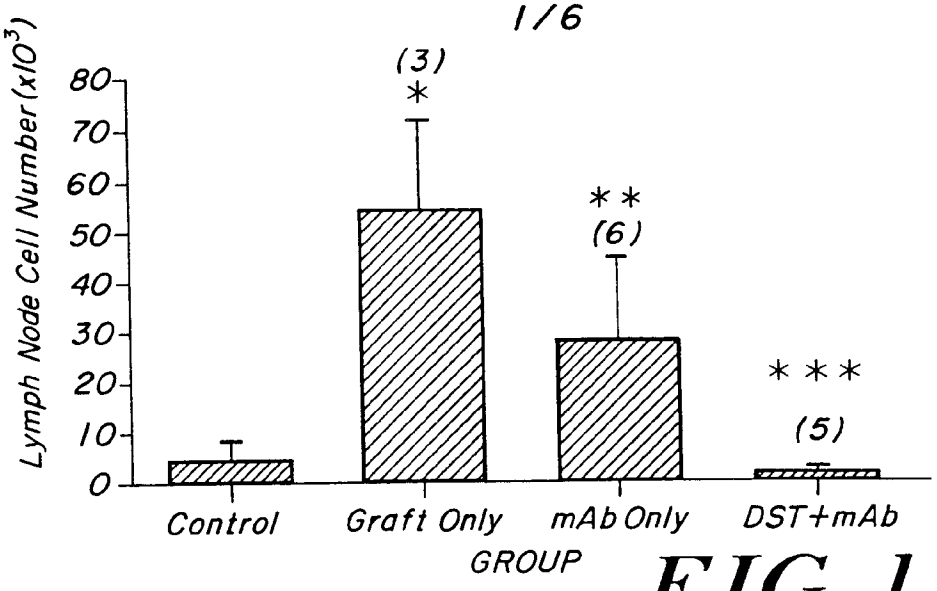

FIG. 1

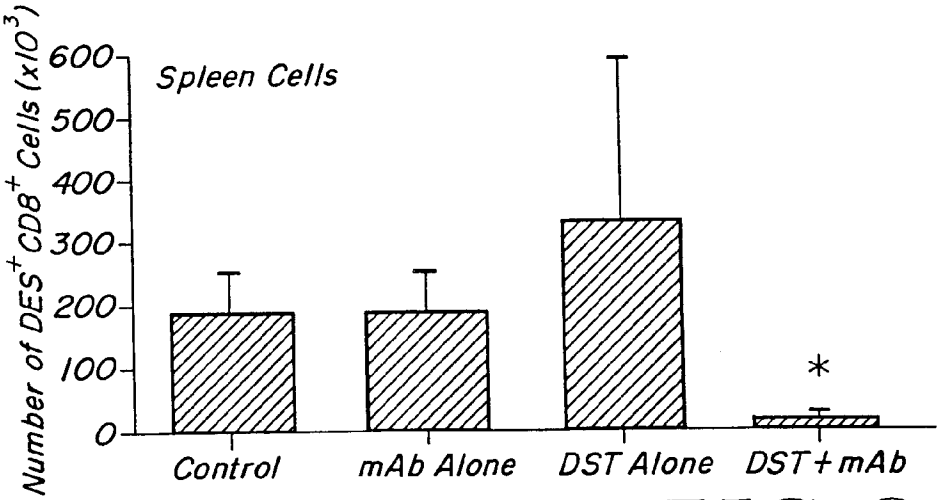

FIG. 2A

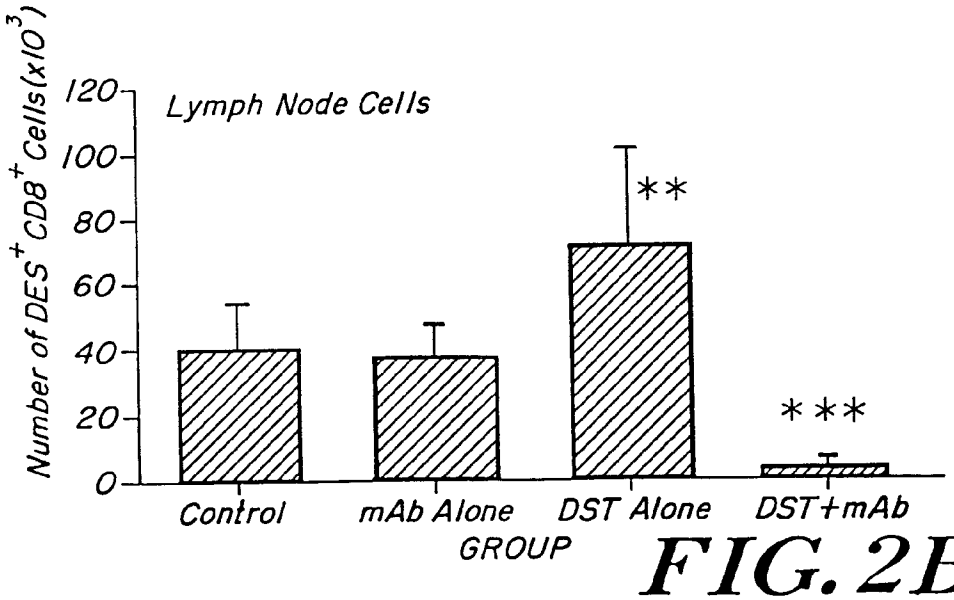

FIG. 2B

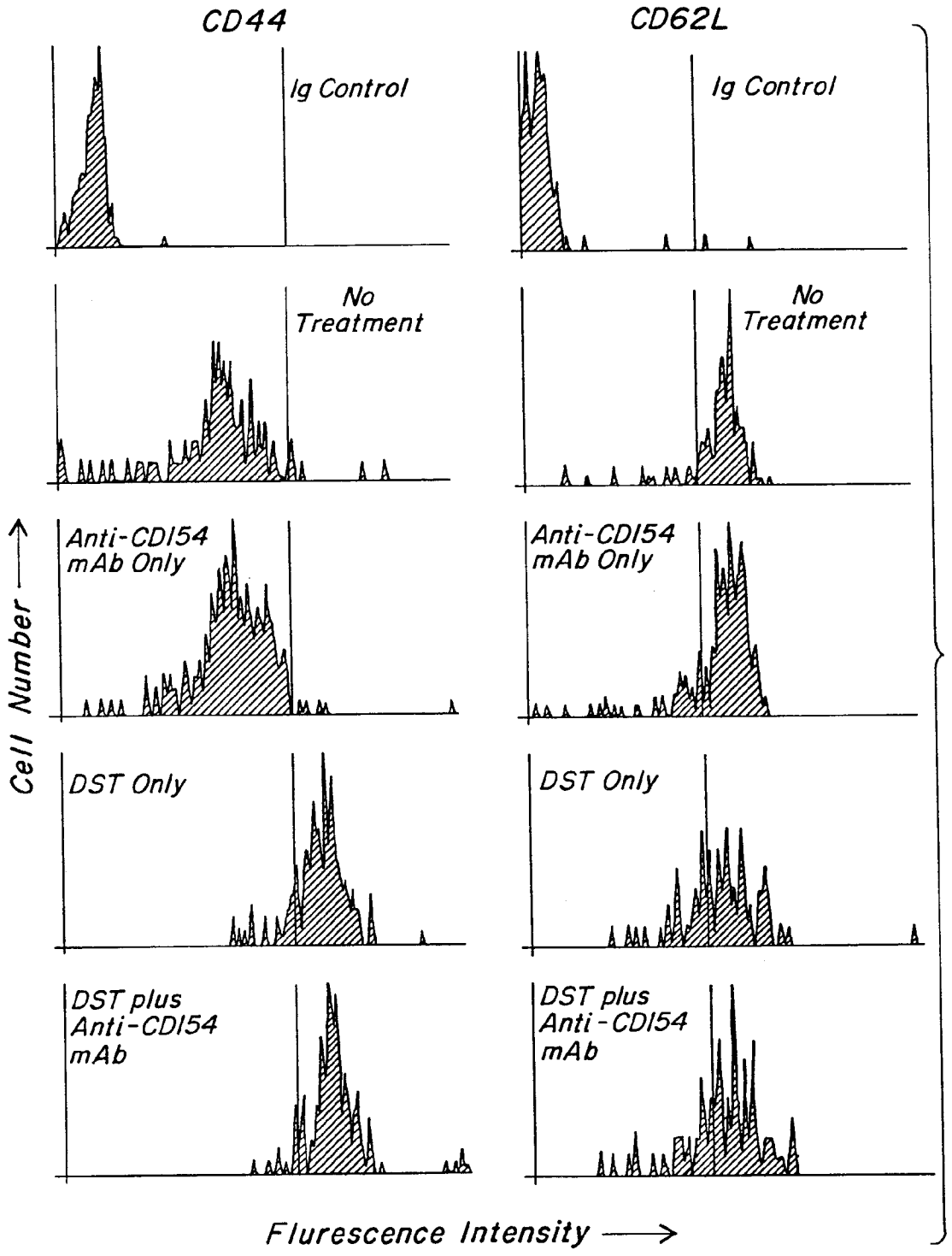
CD44
CD62L
Ig Control
Ig Control
No Treatment
No Treatment
Anti-CD154 mAb Only
Anti-CD154 mAb Only
DST Only
DST Only
DST plus Anti-CD154 mAb
DST plus Anti-CD154 mAb
Cell Number
Flurescence Intensity

FIG. 3

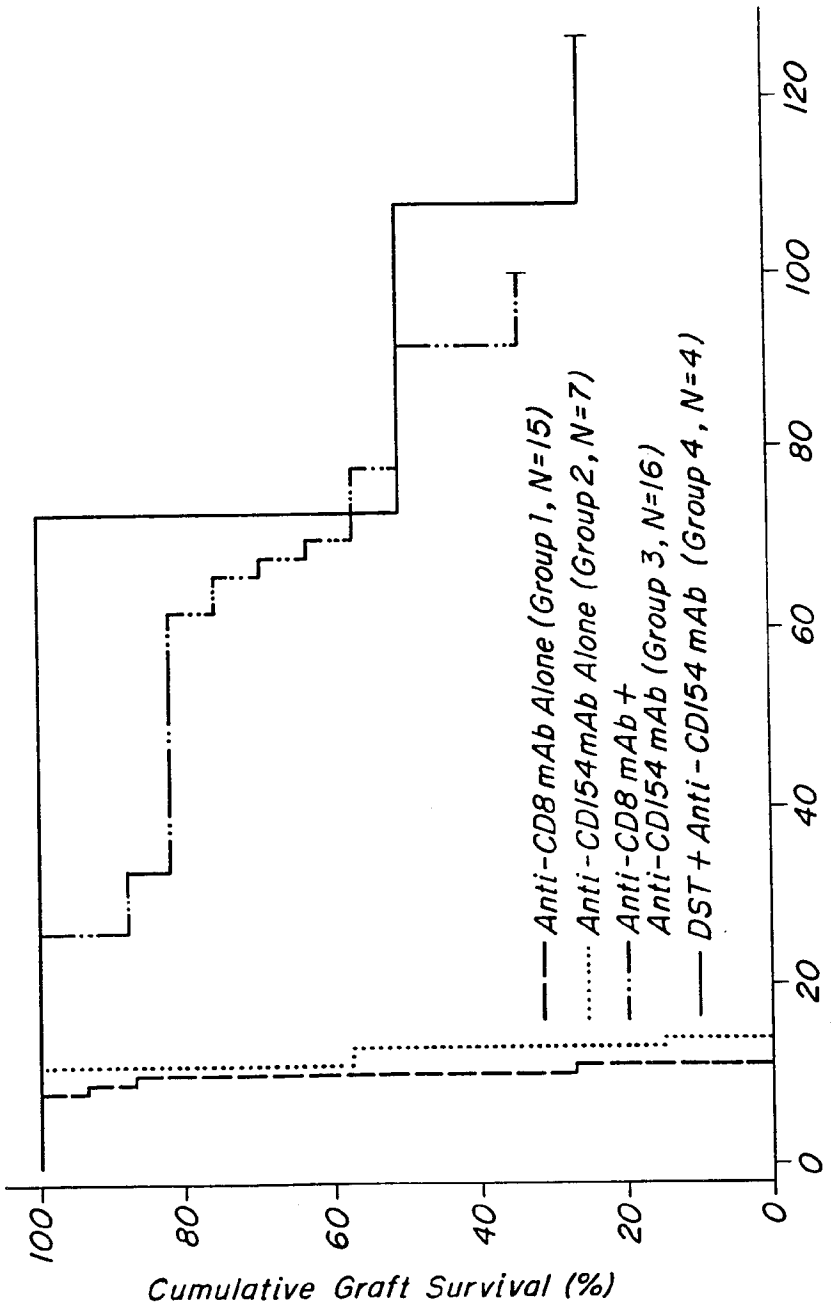
Cumulative Graft Survival (%)
0
20
40
60
80
100
0
20
40
60
80
100
120
Anti-CD8 mAb Alone (Group 1, N=15)
Anti-CD154 mAb Alone (Group 2, N=7)
Anti-CD8 mAb + Anti-CD154 mAb (Group 3, N=16)
DST + Anti-CD154 mAb (Group 4, N=4)

FIG. 4A

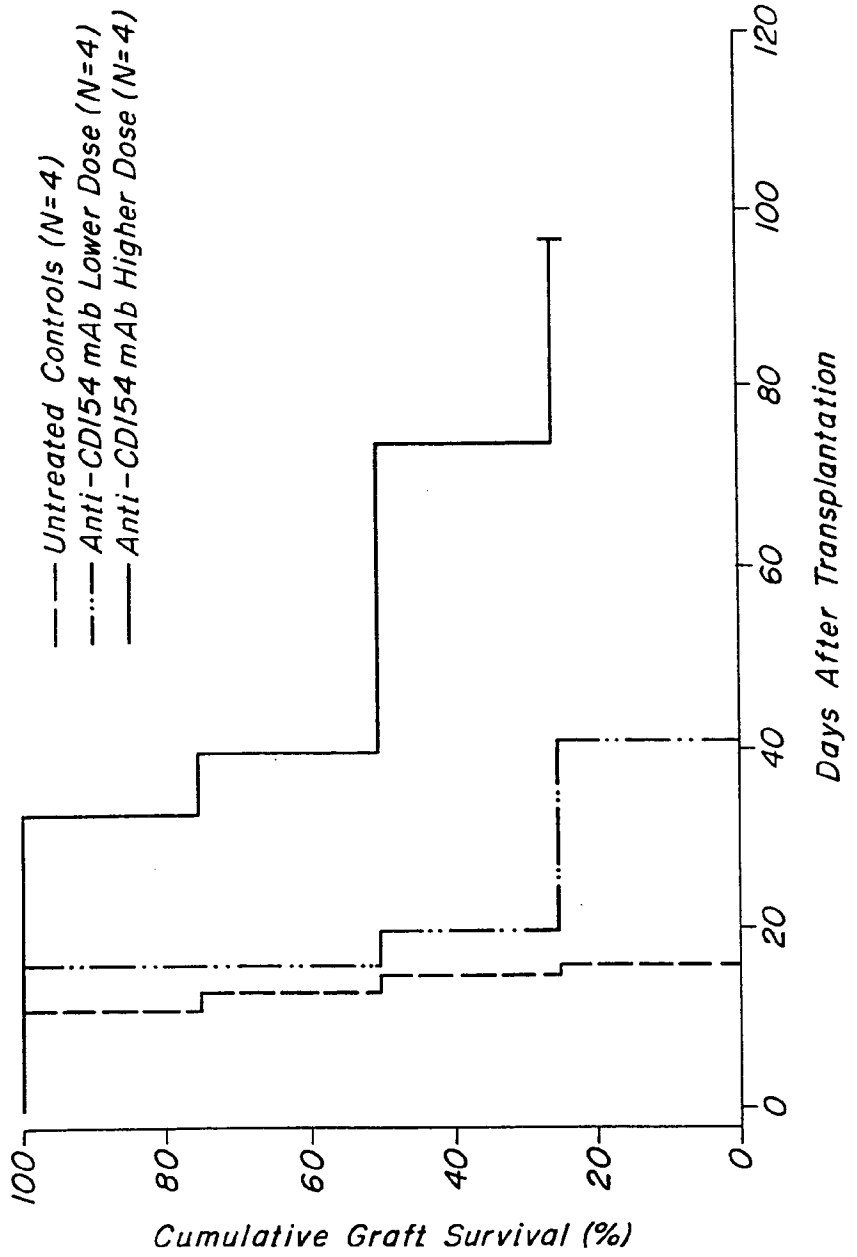
Untreated Controls (N=4)
Anti-CD154 mAb Lower Dose (N=4)
Anti-CD154 mAb Higher Dose (N=4)
Cumulative Graft Survival (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
Days After Transplantation

FIG. 4B

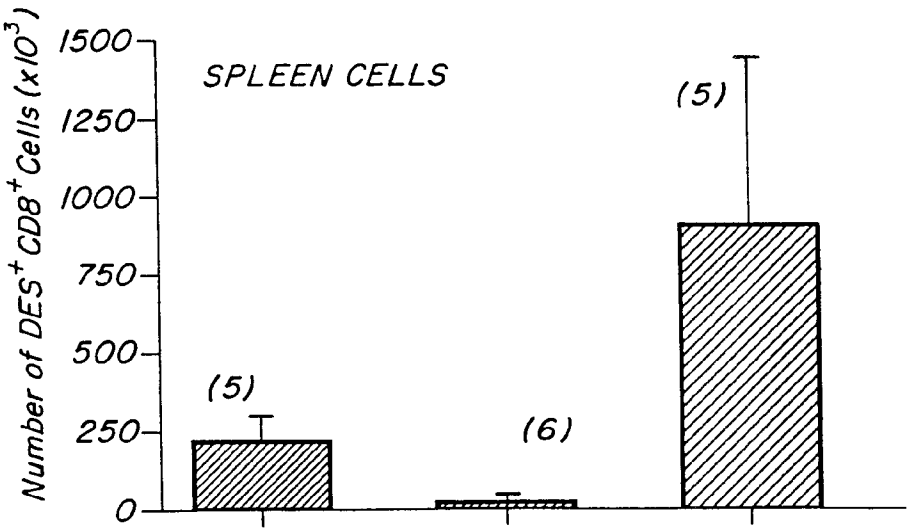
Number of DES+CD8+Cells (x10^3)
1500
1250
1000
750
500
250
0
SPLEEN CELLS
(5)
(6)
(5)

FIG. 5A

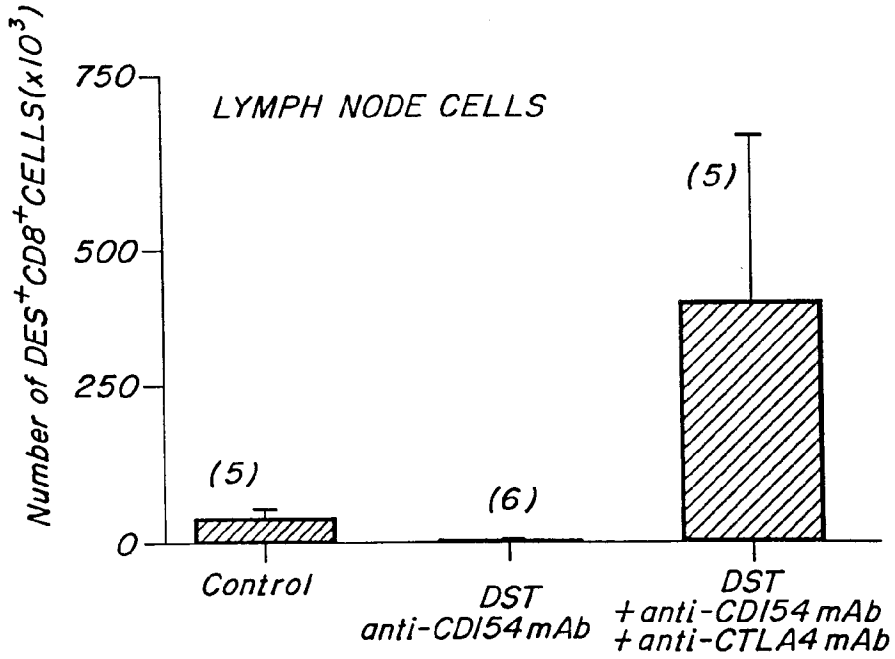
Number of DES+CD8+CELLS(x10^3)
750
500
250
0
LYMPH NODE CELLS
(5)
(6)
(5)
Control
DST
anti-CD154mAb
DST
+anti-CD154mAb
+anti-CTLA4mAb

FIG. 5B

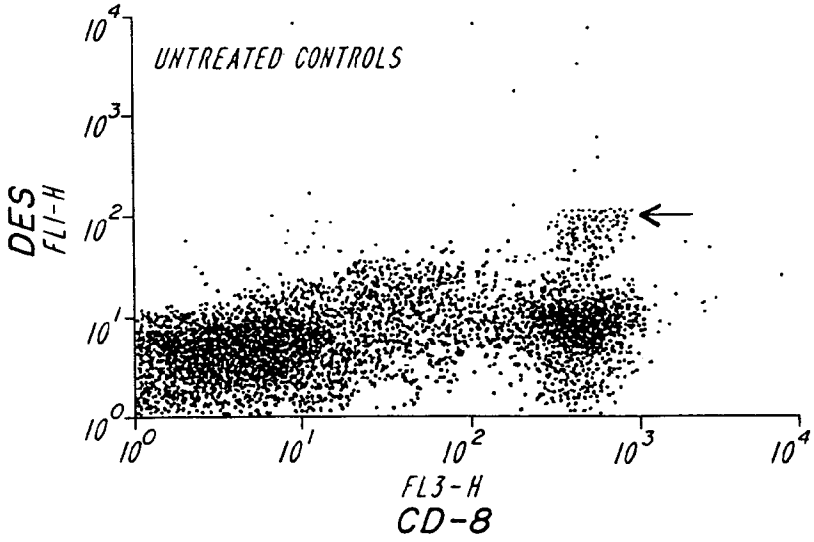

FIG. 6A

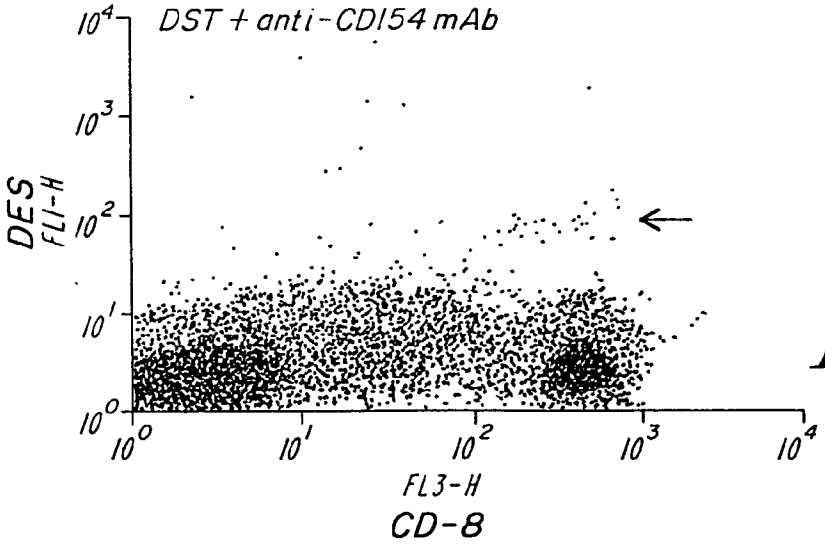

FIG. 6B

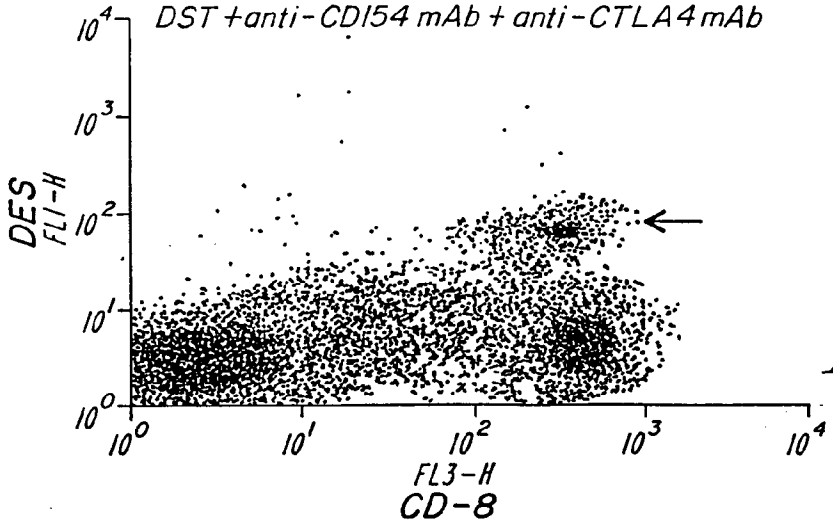

FIG. 6C